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Increased numbers of FoxP3-expressing $CD4^+CD25^+$ regulatory T cells in peripheral blood from dogs with atopic dermatitis and its correlation with disease severity

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Increased numbers of FoxP3-expressing $CD4^+CD25^+$ regulatory T cells in peripheral blood from dogs with atopic dermatitis and its correlation with disease severity

Summary:

Background – Atopic dermatitis (AD) is a common chronic inflammatory skin disease of humans and dogs. Regulatory T cells (Tregs) are essential controllers of immune homeostasis and have been shown to play a key role in human AD, even though frequencies of Tregs in atopic human patients vary greatly. Only two studies have reported Treg numbers in the peripheral blood of dogs with canine AD (CAD).
Objectives – This study aimed to assess the numbers of circulating Tregs in healthy and atopic dogs, and to determine whether Treg numbers correlate with age, sex, disease severity or pre-treatment.
Animals – Client-owned dogs including 14 healthy dogs and 35 dogs with CAD.
Methods – Expression of Tregs in peripheral blood mononuclear cells was evaluated by flow cytometry. Tregs were phenotypically identified as T cells triple positive for CD4, CD25 and FoxP3.
Results – The percentage of circulating $CD4^+CD25^+FoxP3^+$ Tregs in atopic dogs was increased significantly compared to healthy dogs (mean 2.1% versus 1%, $P = 0.002$) and correlated with disease severity (Pruritus Scale: $r = 0.48$, $P = 0.003$; CADESI-04: $r = 0.34$, $P = 0.044$). No significant differences in age or sex were found in either group and pre-treatment had no influence on results for atopic dogs.
Conclusions – Data suggest that, as in humans, $CD4^+CD25^+FoxP3^+$ Tregs may contribute to the pathogenesis of CAD as indicated by an association between Treg frequency and disease severity. Further investigation is required to improve the understanding of the role of Tregs in atopic dogs.

Canine Atopic Dermatitis – Regulatory T Cells – $CD4^+CD25^+FoxP3^+$ Tregs – FoxP3 – Flow Cytometry

Vetsuisse-Fakultät Universität Zürich 2016

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Zusammenfassung:

Hintergrund – Die atopische Dermatitis (AD) ist eine häufige chronisch-entzündliche Hauterkrankung bei Menschen und Hunden. Die regulatorischen T Zellen (Tregs) sind essentielle Kontrolleure der Homöostase des Immunsystems. Es konnte gezeigt werden, dass sie bei der AD des Menschen eine wichtige Schlüsselrolle einnehmen, obwohl die Anzahl der Tregs bei atopischen Menschen stark variieren. Es gibt bisher nur zwei Studien, die über die Anzahl der Tregs im peripheren Blut von Hunden mit caniner AD (CAD) berichten.
Ziele – Das Ziel dieser Studie war die Erhebung der Anzahl zirkulierender Tregs bei gesunden und atopischen Hunden, und festzustellen, ob die Anzahl der Tregs mit dem Alter, dem Geschlecht und der Schwere der Erkrankung oder mit einer Vorbehandlung korrelierten.
Tiere – Hunde im Privatbesitz, davon 14 gesunde Hunde und 35 Hunde mit CAD.
Methoden – Die Exprimierung der Tregs in peripheren mononukleären Zellen des Blutes wurde mittels Flowzytometrie bestimmt. Die Tregs wurden phänotypisch als CD4, CD25 und FoxP3 positiv identifiziert.
Ergebnisse – Der Prozentsatz der zirkulierenden $CD4^+CD25^+FoxP3^+$ Tregs bei atopischen Hunden war im Vergleich zu gesunden Hunden signifikant erhöht (Durchschnitt 2,1% versus 1%, P = 0,002) und korrelierte mit der Schwere der Erkrankung (Juckreizskala: r = 0,48, P = 0,003; CADESI-04: r = 0,34, P = 0,044). Es bestanden in beiden Gruppen keine signifikanten Unterschiede abhängig vom Alter oder vom Geschlecht, auch hatte eine Vorbehandlung keinen Einfluss auf die Ergebnisse bei atopischen Hunden.
Schlussfolgerungen – Die Ergebnisse weisen darauf hin, dass wie beim Menschen $CD4^+CD25^+FoxP3^+$ Tregs an der Pathogenese der CAD teilhaben, was sich durch den Zusammenhang der Anzahl der Tregs und der Schwere der Erkrankung zeigte. Weitere Untersuchungen sind notwendig, um das Verständnis der Rolle der Tregs bei atopischen Hunden zu erweitern.

Canine Atopische Dermatitis – Regulatorische T Zellen – $CD4^+CD25^+FoxP3^+$ Tregs – FoxP3 – Flowzytometrie

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Increased numbers of FoxP3-expressing CD4$^+$ CD25$^+$ regulatory T cells in peripheral blood from dogs with atopic dermatitis and its correlation with disease severity

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Background – Atopic dermatitis (AD) is a common chronic inflammatory skin disease of humans and dogs. Regulatory T cells (Tregs) are essential controllers of immune homeostasis and have been shown to play a key role in human AD, even though frequencies of Tregs in atopic human patients vary greatly. Only two studies have reported Treg numbers in the peripheral blood of dogs with canine AD (CAD).

Objectives – This study aimed to assess the numbers of circulating Tregs in healthy and atopic dogs, and to determine whether Treg numbers correlate with age, sex, disease severity or pre-treatment.

Animals – Client-owned dogs including 14 healthy dogs and 35 dogs with CAD.

Methods – Expression of Tregs in peripheral blood mononuclear cells was evaluated by flow cytometry. Tregs were phenotypically identified as T cells triple positive for CD4, CD25 and FoxP3.

Results – The percentage of circulating CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs in atopic dogs was increased significantly compared to healthy dogs (mean 2.1% versus 1%, $P = 0.002$) and correlated with disease severity (Pruritus Scale: $r = 0.48$, $P = 0.003$; CADESI-04: $r = 0.34$, $P = 0.044$). No significant differences in age or sex were found in either group and pre-treatment had no influence on results for atopic dogs.

Conclusions – Data suggest that, as in humans, CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs may contribute to the pathogenesis of CAD as indicated by an association between Treg frequency and disease severity. Further investigation is required to improve the understanding of the role of Tregs in atopic dogs.

Introduction

Atopic dermatitis (AD) is a common, chronic and relapsing inflammatory skin disease which affects several animal species. Many clinical and pathogenetic similarities are observed between human AD and canine AD (CAD). Numerous studies have shown that AD involves both skin barrier dysfunction and abnormal immunological responses to allergens in genetically susceptible individuals.[1,2] Human AD is associated with Th1/Th2 imbalances and modified immune suppression induced by regulatory T cells (Tregs).[3,4]

Tregs are an unique T-lymphocyte subgroup, representing up to 10% of all CD4$^+$ T cells, and are divided into different subsets which include naturally occurring thymus-derived and peripherally induced Tregs.[5,6] Each of the two subpopulations can be phenotypically identified by surface markers such as CD4 and CD25 as well as the intranuclear transcription factor FoxP3, which define the most studied and most commonly accepted phenotype.[5–7] Tregs are known to prevent excessive immune responses through their immunoregulatory properties and therefore play a leading role in the maintenance of immunological homeostasis.[8,9] Thus, abnormalities in the numbers or function of Tregs have been implicated in the pathogenesis of allergic diseases.[3,10–12]

In humans, several studies have demonstrated altered numbers of circulating Tregs in AD patients compared with healthy controls.[10,13–16] It is likely that Tregs play an important role in CAD as well, given the immunological similarities with its human counterpart. However, very little is known about Tregs in atopic dogs. To the best of the authors' knowledge only three studies[17–19] have attempted to quantify canine Tregs and only one of these evaluated Tregs in the peripheral blood of dogs with spontaneously occurring CAD.[18] There was no difference in circulating Tregs of atopic and healthy dogs at baseline; an increase in the circulating Treg population of dogs with CAD during allergen-specific immunotherapy was reported.[18] The presence of CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs has recently been described in canine skin, although

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numbers were not significantly different between healthy and atopic dogs.[19]

The goal of the present study was to assess the quantity of circulating Tregs in dogs with CAD. Using three-colour flow cytometric analysis, we specifically evaluated the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs in peripheral blood mononuclear cells (PBMC) of atopic dogs compared to healthy individuals. We hypothesized that a statistically significant difference in Treg frequency would be detected between the groups, providing new insight into the potential role of Tregs in the pathogenesis of CAD. We investigated the relationship between Treg frequency and age, sex and recent immunomodulatory treatment. We also explored whether Treg frequencies correlate with clinical features of CAD as have been described for human atopic diseases, such as the course or severity of disease.[10,16,20]

Materials and methods

Animals and clinical evaluations

Thirty five atopic and 14 healthy client-owned dogs greater than 6 months of age were included in the study. Informed consent was obtained from each dog owner before the study and all procedures were approved by the Cantonal Veterinary Office of Zurich and conducted in accordance with guidelines established by the Animal Welfare Act of Switzerland. Control samples were obtained from healthy dogs with no histories of allergic skin diseases. Vaccination within the previous month, treatment with immunomodulatory agents, dermatitis of any type and diseases known to affect the immune system were exclusion criteria for healthy controls.

Dogs with CAD were recruited from the dermatology unit at the Vetsuisse Faculty, University of Zurich. All atopic dogs had been affected for a minimum of 3 months prior to recruitment and were selected based on their medical histories, clinical features and fulfilment of at least five of eight criteria.[21] Differential diagnoses such as ectoparasitic and infectious dermatoses were excluded by diagnostic investigations including skin scrapings, cytological examination, bacterial and/or fungal culture when deemed appropriate, and lack of response to therapeutic trials when needed.

Although adverse reaction to foods was not considered to be an exclusion criterion, all subjects underwent a dietary elimination trial of 6–8 weeks duration using novel ingredients or hydrolysed commercial diets. Dietary re-challenge was performed in cases that demonstrated improvement on the diet trial. None of the dogs responded completely to this procedure, but seven demonstrated partial improvements associated with dietary restriction.

Upon enrolment, severity of CAD was assessed using a validated visual analog scale (VAS) for pruritus[22] and a previously validated scoring system (Canine Atopic Dermatitis Extent and Severity Index, version 4; CADESI-04).[23] Screening for environmental allergen sensitivities was performed in all atopic dogs and consisted of intradermal testing carried out with Greer allergens (Greer Veterinary Laboratories, Lenoir, NC, USA) and/or allergen-specific IgE determination by ELISA (HESKA inc., Fribourg, Switzerland). All subjects included in the study demonstrated positive results with at least one of the two tests. Positive reactions were evaluated and found to be compatible with the clinical history of all of the dogs.

Clinical data collected included age, sex, course of disease (perennial versus recurrent/seasonal signs), severity of CAD (pruritus, CADESI-04) and recent treatment with immunomodulatory drugs (glucocorticoids, ciclosporin and/or oclacitinib).

Immunomodulatory drugs were discontinued in the majority of the subjects for a minimum of 2 weeks prior to blood sampling and intradermal testing. However, nine subjects were still receiving immunomodulatory drugs at the time of sampling due to the severity of their clinical signs. Such drugs had been reduced to the minimum doses necessary during the two preceding weeks (see Table 1 for details on agent and dosage). None of the subjects had received allergen-specific immunotherapy. Oral antimicrobial drugs and topical antibacterial or antifungal agents were permitted up to the time of testing. Serological and intradermal tests were performed after microbial and parasitic conditions had been resolved, but when clinical signs of cAD were still present.

Blood sampling and separation of PBMC

Approximately 2 mL of whole blood was collected from each control or atopic dog by jugular or antebrachial cephalic venepuncture and transferred into EDTA Vacuette tubes (Greiner Bio-One, Kremsmünster, Austria). Blood samples were stored at room temperature (RT) and processed within 24 h after collection.

PBMC were isolated from whole blood using a standard density gradient centrifugation by Histopaque-1077 g/mL (Sigma-Aldrich, St. Louis, MO, USA) as described elsewhere.[10,13] Anticoagulated blood was layered onto Histopaque-1077, followed by centrifugation (700***g***) for 25 min at RT. The layer of PBMC was collected from the interface, washed and re-suspended in HBSS (Hank's balanced salt solution, Gibco, Life Technologies; Carlsbad, CA, USA) supplemented with 3% inactivated fetal calf serum (FCS) to establish cell count and viability. The cell viability was determined by flow cytometry with Guava ViaCount Flex (Merck Millipore; Billerica, MA, USA) and ranged from 95 to 99%.

Cell staining and flow cytometric analysis of Tregs

A typical Treg phenotype based on the simultaneous expression of CD4, CD25 and FoxP3 was analysed by flow cytometry according to protocols described previously.[7,24–27]

In order to detect cell surface (CD4, CD25) and intranuclear (FoxP3) markers, freshly isolated PBMC were stained with a combination of dog-specific or cross-reactive fluorochrome-conjugated monoclonal antibodies (mAbs) as follows: anti-canine CD4 fluorescein isothiocyanate (FITC) (clone YKIX302.9, AbD Serotec; Raleigh, NC, USA), anti-canine CD25 phycoerythrin (PE) (clone P4A10, eBioscience; San Diego, CA, USA) and cross-reactive anti-mouse/rat FoxP3 eFluor660 (clone FJK-16s; eBioscience). Appropriate isotype controls were performed for each experiment. FoxP3 staining was conducted following the manufacturer's instructions using the permeabilization buffer and fixation/permeabilization reagents provided (FoxP3 Staining Set; eBioscience). In brief, freshly isolated PBMC were suspended in 50 μL of HBSS supplemented with 3% FCS (HBSS/3% FCS) and incubated directly with 5 μL of both anti-CD4 FITC and anti-CD25 PE for 30 min at 4°C in the dark. The optimal working dilutions of mAbs were determined on a previously performed antibody titration. Following surface staining, cells were washed with HBSS/3% FCS for 5 min at RT (400***g***). The pellet was then re-suspended in FoxP3 fixation/permeabilization working solution and left at 4°C for 30 min in the dark. Cells were washed consecutively with permeabilization buffer and probed for the intranuclear marker FoxP3 with anti-FoxP3 eFluor660 at the concentration recommended by the manufacturer (0.5 μg per 10^5 to 10^8 cells), then allowed to interact for 30 min at 4°C in the dark. Subsequently, the stained cells were washed twice with permeabilization buffer and re-suspended in 200 μL HBSS/3% FCS for flow cytometric analysis.

Flow cytometric acquisition was performed using a Guava easyCyte 8HT Flow Cytometer (Merck Millipore) and interpreted using the corresponding software GuavaSoft 2.5. In each sample, 10,000 events were analysed. Tregs in PBMC were identified as T cells triple-positive for CD4, CD25 and FoxP3. Initially, the fraction of lymphocytes was selected by typical forward- and side-scattered properties[28] and used for further analysis. Cells positive for CD4 were then gated on a FITC histogram and analysed for CD25 and FoxP3. The number of Tregs was expressed as a percentage of the total $CD4^+$ T cell population.

Table 1. Atopic dogs: clinical characteristics and percentage of canine regulatory T cells (Tregs)

Number	Sex	Age*	Diagnosis	Course of disease†	Treatment‡	CADESI-04	Pruritus	% $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs	% $CD4^+$ $FoxP3^+$ T cells
1	mn	7	CAD	1	0	14	7	2.38	7.37
2	f	1	CAD	1	0	4	7	3.61	9.49
3	fn	8	CAD	1	0	18	5	4.06	9.46
4	fn	7	CAD/FA	1	0	2	3	2.02	3.98
5	mn	4	CAD	1	0	5	4	2.24	6.72
6	mn	6	CAD	0	0	25	3	2.66	5.10
7	f	1	CAD	1	1	7	3	2.70	7.03
8	fn	4	CAD	0	1	8	4	2.52	13.13
9	fn	6	CAD/FA	0	0	21	5	0.76	2.98
10	m	4	CAD	1	2	35	3	1.95	7.55
11	m	2	CAD/FA	1	3	19	8	3.55	9.48
12	mn	3	CAD	1	0	36	9	3.44	16.56
13	m	4	CAD	1	0	27	5	0.33	1.37
14	m	6	CAD	0	0	14	2	0.83	1.72
15	m	6	CAD	1	0	10	5	1.05	9.88
16	m	1	CAD	1	0	14	6	1.49	7.63
17	f	2	CAD	0	0	9	2	2.14	10.33
18	fn	2	CAD	0	0	9	1	1.27	8.04
19	fn	10	CAD	1	0	24	2	1.06	2.27
20	f	1	CAD/FA	1	0	10	10	0.93	3.57
21	mn	9	CAD	0	0	8	2	0.68	1.75
22	mn	4	CAD	1	1	19	4	0.92	3.13
23	fn	3	CAD	1	3	16	4	0.67	2.74
24	m	4	CAD/FA	0	0	16	2	0.65	3.02
25	mn	4	CAD	1	0	10	8	2.80	10.58
26	fn	7	CAD	1	3	4	8	4.79	27.25
27	f	6	CAD/FA	1	0	22	5	1.62	2.36
28	mn	3	CAD	1	0	62	10	2.38	29.19
29	m	8	CAD	1	0	89	7	3.65	30.29
30	m	1	CAD/FA	1	0	26	7	1.09	9.60
31	mn	2	CAD	1	0	10	1	2.02	8.58
32	mn	7	CAD	1	0	79	8	3.53	19.56
33	mn	3	CAD	1	1	117	10	3.75	20.56
34	m	2	CAD	1	1	35	6	2.32	5.80
35	m	1	CAD	1	0	11	6	1.62	2.84

CAD, canine atopic dermatitis; FA, food allergy; CADESI-04, Canine Atopic Dermatitis Extent and Severity Index, version 4; f, female; m, male; n, neutered.

*Rounded.

†0 = seasonal/recurrent, 1 = permanent.

‡Immunomodulatory treatment during the 2 weeks preceding the test: 0, none; 1, prednisolone 0.5 mg/kg every other day (eod); 2, ciclosporin 5 mg/kg eod; 3, oclacitinib at the recommended dosage (0.4–0.6 mg/kg) once daily.

Data analyses

Tregs were defined as $CD4^+$ $CD25^+$ $FoxP3^+$ cells for the purposes of this study. However, data on the population of $CD4^+$ $FoxP3^+$ cells were also collected for comparison, because the $CD4^+$ $FoxP3^+$ phenotype has been used for Treg characterization in several other studies.[7,18,25–27] Correlation was determined between the two populations.

The initial analyses compared the proportion of Tregs in atopic and healthy dogs, and evaluated for correlation with age and sex. Subsequent analyses were restricted to the atopic group. The potential effects of disease course and pre-treatment with immunomodulatory drugs on Treg numbers were evaluated. Dogs with recurrent/seasonal and perennial clinical signs, as well as treated and untreated dogs, were compared. Correlations were also calculated between Treg proportions and disease severity as assessed by CADESI-04 and the VAS. Finally, the impact of concurrent immunomodulatory treatments (glucocorticoids, ciclosporin and oclacitinib) on these correlations was investigated.

Statistical methods

Statistical analyses were performed using SPSS software v22.0 (IBM Corp; Armonk, NY, USA). The means of the datasets, which were not normally distributed as shown by the Kolmogorov–Smirnov test, were compared using the nonparametric Mann–Whitney U-test for independent samples. Correlation between populations of $CD4^+$ $FoxP3^+$ and $CD4^+$ $CD25^+$ $FoxP3^+$ cells was evaluated using Spearman's test. Correlations between normally distributed quantitative variables were analysed using Pearson's r-test because a linear relationship between parameters was assumed. Statistical significance was defined as $P < 0.05$.

Results

Animals and clinical data

Fourteen healthy (male/female ratio: 7/7; mean age: 5.5 years) and 35 atopic (m/f ratio: 21/14; mean age: 4.3 years) dogs were included. Table 1 shows signalment and clinical data for the dogs with CAD. Most subjects enrolled in the study demonstrated mild AD (CADESI-04 index 10–34: 19 dogs) or AD in remission (index <10: 9 dogs) according to the CADESI-04 severity categories.[23] A few subjects demonstrated moderate (index 35–59: 3

dogs) or severe (index ≥60: 4 dogs) disease. In total, index scores ranged from 2 to 117 (mean = 24) on the CADESI-04 scale which has a maximum score of 180.

Proportions of CD4+ CD25+ FoxP3+ and CD4+ FoxP3+ Tregs in PBMC

The proportion of circulating Tregs was defined as a percentage of the total CD4+ T-cell population. The gating strategy utilized and representative dot plots of healthy and atopic dogs are presented in Figure 1. In the PBMC of healthy individuals, Tregs defined as CD4+ CD25+ FoxP3+ ranged from 0.22 to 2.48% (mean 1%). In atopic dogs the same population ranged from 0.33 to 4.79% (mean 2.1%; Table 1). Statistically this difference was highly significant ($P = 0.002$; Figure 2). It is worth noting that despite this high level of significance, Treg proportions from both study populations overlapped.

The proportion of CD4+ FoxP3+ cells was also significantly greater in dogs with CAD (range 1.37–30.29%, mean 9.2%; Table 1) than healthy dogs (range 0.94–6.01%, mean 3.4%; $P = 0.005$). The proportions of CD4+ FoxP3+ and CD4+ CD25+ FoxP3+ cells were strongly correlated ($r = 0.76$, $P < 0.001$).

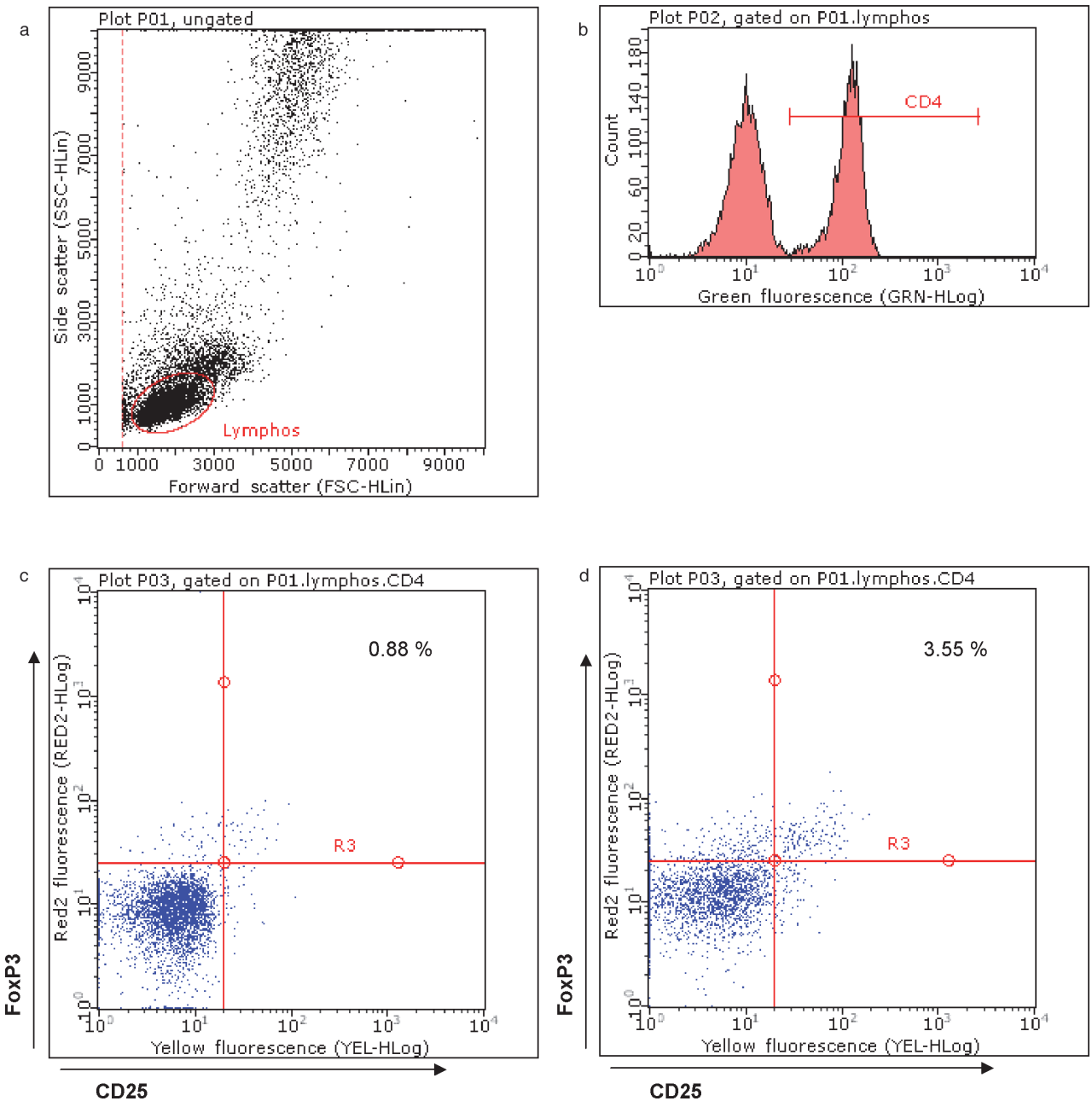

Figure 1. Gating strategy and flow cytometric evaluation of canine regulatory T cells (Tregs). (a) Peripheral blood mononuclear cells (PBMC) of healthy and atopic dogs were stained with monoclonal antibodies against CD4, CD25 and intranuclear FoxP3. Initially the lymphocyte population was gated based on side scatter (SSC) and forward scatter (FSC) distribution (gate 1). (b) Then, CD4+ cells were identified in a fluorescein isothiocyanate (FITC) histogram (gate 2) and used in subsequent analysis for CD25 and FoxP3 expression (dot plot CD25 versus FoxP3). Representative dot plots are shown from a healthy dog (c) and a dog with atopic dermatitis (AD) (d). The numbers in the right upper quadrants represent the respective percentages of CD4+ CD25+ FoxP3+ Tregs in gated CD4+ T-cell populations.

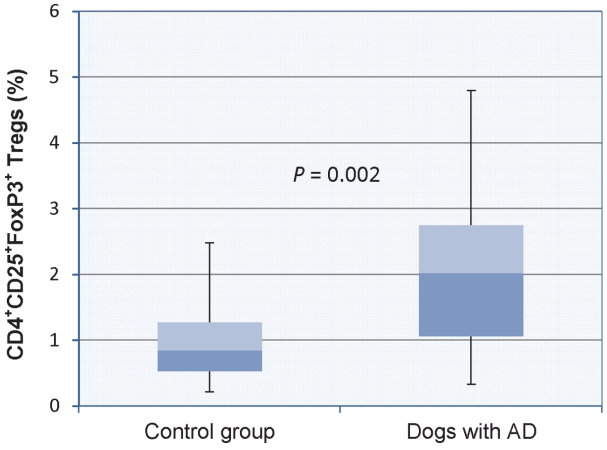

Figure 2. Comparison of percentage of canine regulatory T cells (Tregs) between atopic and healthy dogs. The percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ T_{regs} in gated $CD4^+$ T cells was significantly increased (mean 2.1% versus 1%) in peripheral blood of dogs with atopic dermatitis (AD) ($n = 35$) compared with healthy controls ($n = 14$). Results are presented as box plots with minimum, maximum, median and 25th/75th percentiles. A nonparametric Mann–Whitney U-test was used for comparison between both groups ($P = 0.002$).

Correlation of Treg percentage with clinical features

There were no statistical differences in Treg proportions within the atopic and healthy groups in regard to age and sex, nor was there an association with seasonality of clinical signs or immunomodulatory drug therapy within the atopic group. However, there was an association with disease severity: proportions of Tregs showed a moderate positive correlation with pruritus ($r = 0.48$, $P = 0.003$) and, to a lesser extent, with CADESI-04 scores ($r = 0.34$, $P = 0.044$). When dogs that had received immunomodulatory drug therapy were removed from the analysis, positive correlations were maintained: $r = 0.39$ for pruritus ($P = 0.047$) and $r = 0.41$ for CADESI-04 ($P = 0.038$) (Figure 3).

Most of the atopic dogs had mild AD ($n = 19$) or AD in remission ($n = 9$) and only few showed moderate ($n = 3$) or severe ($n = 4$) category scores for the disease. We also evaluated Treg percentage and CADESI-04 scores in relation to the severity category. No significant relationship was observed between severity groups.

Discussion

In the present study it was shown that the proportions of FoxP3-expressing $CD4^+$ $CD25^+$ Tregs in the peripheral blood of dogs with CAD was significantly increased compared to healthy dogs. Furthermore, Treg levels correlated with CADESI-04 and the VAS, suggesting that Tregs are associated with disease severity.

Circulating Tregs have been evaluated in healthy dogs in several previous works indicating that canine Treg levels range from 0.7 to 7.2% (Table 2).[7,18,24–27,29,30] The results of these studies are sometimes difficult to compare because of methodological differences such as variation in antibodies, gating strategies and Treg definitions ($CD4^+$ $CD25^+$, $CD4^+$ $FoxP3^+$ or $CD4^+$ $CD25^+$ $FoxP3^+$ cells). Currently, Tregs in dogs are generally defined by the simultaneous expression of the markers CD4, CD25 and FoxP3.[7,19,29] In order to compare our results with those of previously published studies,[7,18,25–27,29,30] we also analysed populations of $CD4^+$ $FoxP3^+$ cells. In our study, the frequency of these two populations of Tregs in healthy dogs was generally consistent with the corresponding values in previous works (Table 2).

We also found that the proportion of $CD4^+$ $CD25^+$ $FoxP3^+$ cells was markedly lower than the $CD4^+$ $FoxP3^+$ cell population, although the correlation between the two populations was strong and consistent with that demonstrated in previous studies.[7,26,29] In our dataset only 30% of $CD4^+$ $FoxP3^+$ cells were also $CD25^+$, which is in accordance with a previous study.[26] The difference between the two populations suggests that the use of triple staining may be viewed as a more precise definition of Tregs. However, this observation may also raise the question as to what type of cells the $CD25^-$ $CD4^+$ $FoxP3^+$ population truly represents. There are two possible explanations. First, they could constitute activated nonregulatory T cells because both CD25 and FoxP3 can be expressed in naive $CD4^+$ T cells upon activation.[31] Second, they could represent Tregs in an "inactive" state. It has been demonstrated that CD25 on Tregs is fully labile, thus $CD4^+$ $CD25^-$ $FoxP3^+$ cells may be able to act as a peripheral reservoir for "active" $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs upon immune activation.[32]

Our results in dogs with CAD may be compared with two prior studies. One study found that the percentage of $CD4^+$ $FoxP3^+$ Tregs in healthy individuals was similar to those in our study, but in contrast to our results, reported that atopic dogs did not differ from healthy dogs.[18] The second study was carried out on experimentally challenged atopic beagles in which Tregs were defined as $CD4^+$ $CD25^+$.[17] This study reported results similar to our atopic group. To the best of the authors' knowledge, our study is the first to compare Tregs in the peripheral blood of healthy and atopic dogs by using simultaneous triple-positive cells to define the Treg phenotype. Our results mirror those of numerous reports in humans where increased numbers of circulating Tregs have been described for AD patients compared with healthy controls.[10,14–16] Two of these studies also demonstrated that Tregs correlate with disease severity.[10,16] Others detected decreased[13] or similar[33] Treg numbers in AD patients. The reasons for these inconsistent findings remain speculative, but may be associated with the use of variable definitions of Tregs and the existence of different Treg subsets, such as thymus derived (tTregs) or peripherally generated (pTregs) cells.[34] The various results may also be related to age differences, course of disease, stage of the immune response, disease severity or concurrent/prior therapies.

Human studies have indicated that Treg numbers decline with age and that lifelong changes in Treg cell subsets may be observed.[31,35] In three studies evaluating mainly adult patients with AD (the mean ages were 27, 27.1 and 42 years), increased numbers of circulating Tregs were demonstrated compared to healthy controls.[10,14,15] However, one study enrolling children (mean age 7.8 years) showed significantly lower frequencies of Tregs in atopic patients.[13] Our study did not demonstrate

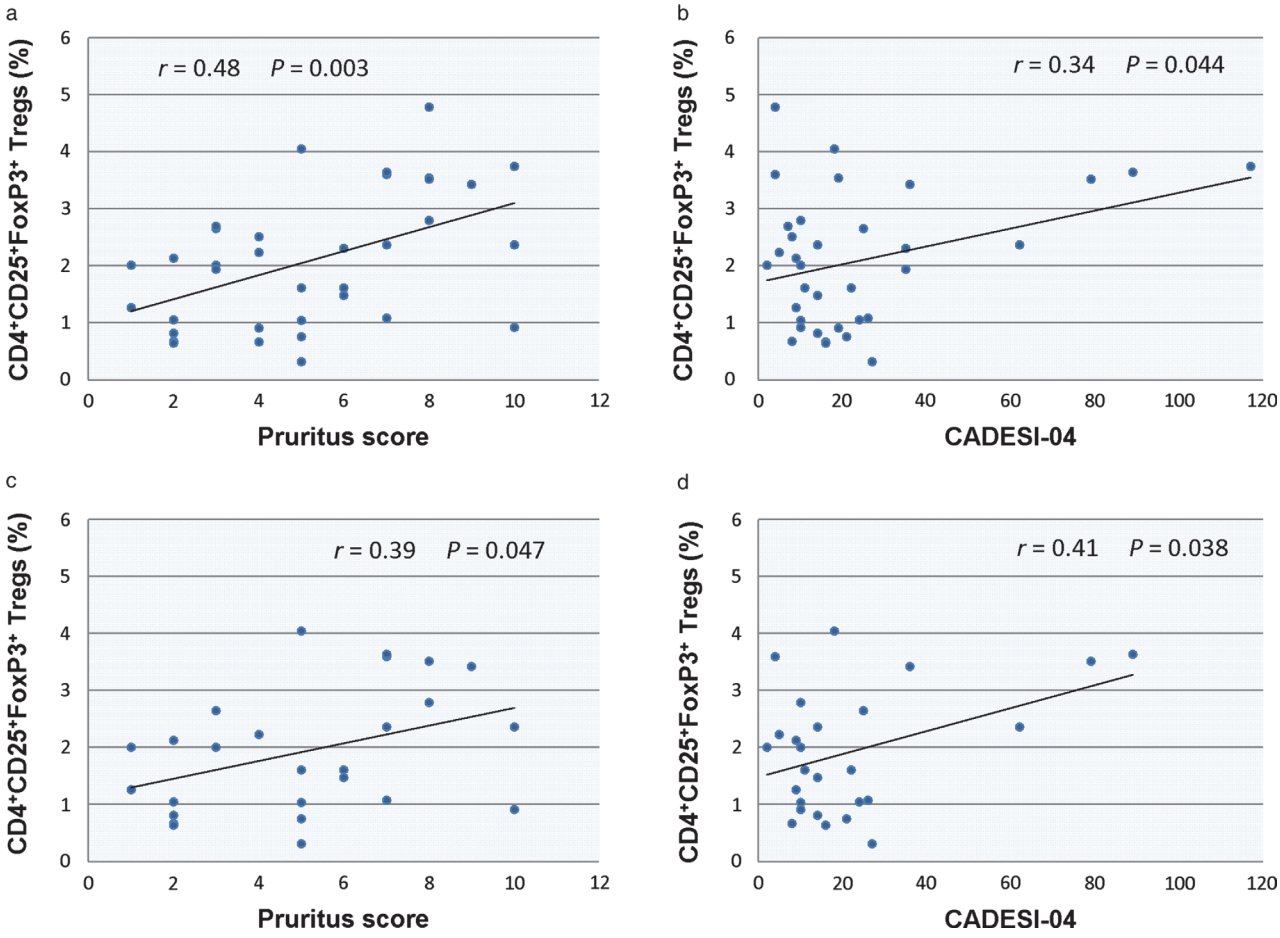

Figure 3. Correlation between percentage of canine regulatory T cells (Tregs) of atopic dogs and their disease severity. (a) The percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs in gated $CD4^+$ T cells of dogs with atopic dermatitis (AD) (n = 35) was correlated positively with a Visual Analog Scale for pruritus (r = 0.48, P = 0.003) and (b) to a minor degree, with Canine Atopic Dermatitis Extent and Severity Index (CADESI)-04 scores (r = 0.34, P = 0.044). (c, d) The removal of dogs treated with immunomodulatory drugs (n = 9) from the group resulted in modified correlations (c) between Tregs and pruritus (r = 0.39, P = 0.047), and (d) between Tregs and CADESI-04 (r = 0.41, P = 0.038). Pearson's r-test was used for correlation analyses.

Table 2. Percentage of canine regulatory T cells (Tregs) in peripheral blood of dogs – comparison of published studies

Year of publication	Reference no.	Treg definition	No. of healthy/atopic	% Tregs in healthy dogs	% Tregs in atopic dogs
2007	25	$CD4^+$ $FoxP3^+$	10/0	4.3	
2008	18	$CD4^+$ $FoxP3^+$	25/53	4.84	4.94
2009	30	$CD4^+$ $FoxP3^+$	9/0	4.04	
2009	27	$CD4^+$ $FoxP3^+$	4/0*	1.08–2.65	
2009	17	$CD4^+$ $CD25^+$	0/6†		1.2–2.1
2010	7	$CD4^+$ $FoxP3^+$	39/0	2.63**	
		$CD4^+$ $CD25^+$		1.98**	
		$CD4^+$ $CD25^+$ $FoxP3^+$		0.98**	
2010	24	$CD4^+$ $CD25^+$	2/0‡	7.2	
2011	29	$CD4^+$ $FoxP3^+$	11/0§	2.8**	
		$CD4^+$ $CD25^+$ $FoxP3^+$		0.8**	
2011	26	$CD4^+$ $FoxP3^+$	12/0¶	3.4	
		$CD4^+$ $CD25^+$ $FoxP3^+$	5/0¶	0.7	
2015	This study	$CD4^+$ $FoxP3^+$	14/35	3.4	9.2
		$CD4^+$ $CD25^+$ $FoxP3^+$		1	2.1

The mean percentage of Tregs is given, unless otherwise indicated.
*Specific pathogen-free dogs.
†Experimental model, before and after challenge, previously sensitized beagles.
‡Exclusively beagles and beagle mixed breeds, immunomagnetically selected with CD3.
§Exclusively beagles.
¶Exclusively greyhounds and beagles aged between 12 and 30 months.
**Median.

a significant correlation with age, but this might be related to the fact that the relative age of onset of AD is greater in dogs than in people.

Besides age, the course of disease may have an impact as human patients with persistent allergic airway disease had more Tregs than those with intermittent disease.[20] In our study we did not find a statistical difference in Treg proportions between CAD subjects with seasonal/recurrent and perennial clinical signs. However, the possibility that the high proportion of Tregs present in our cohort could be related to the chronic nature of the disease cannot be excluded, and it is possible that the proportion would be different in cases of more acute onset.

It is also possible that Treg populations change during AD development, depending on the stage of the immune response and possibly related to a dynamic redistribution between skin and peripheral blood. One study analysed peripherally circulating Tregs in humans with AD at intervals of 1–2 months and demonstrated decreases in Treg frequency as skin lesions improved, suggesting migration to the affected skin.[10] However, we are not aware of existing data to support a similar hypothesis in the dog. It would be interesting to compare canine Treg populations in PBMC and inflamed skin, and to sequentially track these proportions during AD development.

Our study demonstrated a moderate but statistically significant correlation between Treg numbers and disease severity, which has also been observed in several studies of atopic humans.[10,16,20,36] There are several possible explanations for this apparent paradox. First, the higher proportion of Tregs might represent a subset of pTregs generated by conversion from activated effector or memory $CD4^+$ T cells[5] during repeated or prolonged allergen stimulation and skin inflammation as part of the immune response. It has been demonstrated in humans that in addition to tTregs, the phenotype $CD4^+$ $CD25^+$ $FoxP3^+$ can also be adopted by pTregs;[6] however, it is unknown whether this phenomenon occurs in dogs. It is also possible that high numbers of $CD4^+$ $CD25^+$ $FoxP3^+$ cells represent either tTregs and are a consequence of proliferated pre-existing tTregs or upregulated conventional T cells with a Treg phenotype,[5] but without regulatory function. This latter option would also explain the persistence of the allergic disease despite the upregulation of Tregs in our canine subjects.

Indeed, it remains unclear why the increased Tregs in our cohort appear to have no protective effect on clinical signs. Apart from the aforementioned hypotheses, a functional insufficiency of Tregs as demonstrated in allergic asthmatic children[37] could be postulated. However, other studies have found no evidence of an impaired suppressive activity of Tregs in human allergic diseases,[15,20,33,36] even when (in accordance with our results) high Treg numbers were found.[15] Interestingly, when dogs treated with immunomodulatory drugs were removed from the analysis, the correlation between CADESI-04 score and Treg numbers improved. A possible explanation could be that treated dogs have a lower index of severity but that high Tregs are still present in the circulation. However, previous work in humans suggests that Tregs rapidly decrease in the face of clinical improvement.[10,38] Alternatively, immunomodulatory treatment may increase the number of Tregs which, in turn, contribute to the improvement of the severity index. In asthmatic humans, glucocorticoids have been shown to increase FoxP3 expression[39] and Treg percentages.[40] However, we did not detect any significant difference in the proportions of Tregs between treated and untreated atopic dogs.

The variable findings between studies may imply that the role of Tregs in AD is complex and that the pathogenetic mechanisms of Tregs are possibly not the same in all allergic patients. Functional and/or quantitative differences are just as conceivable as the possibility that different Treg subtypes or single populations could be affected. The great overlap between both the healthy and atopic group might be an indication not only that the absolute Treg number plays a role, but also the individual ratio of Tregs to nonregulatoy (effector) T cells can in every allergic patient.

In summary, our preliminary study demonstrates that the proportion of circulating Tregs is increased in atopic dogs, and correlates with severity. These findings suggest that Tregs may be involved in the pathogenesis of CAD. Additional studies will be necessary to determine which subtypes of Tregs are involved, whether they are also modified in acute cases (at the onset of clinical disease) and if increased numbers are associated with high expression of regulatory cytokines such as IL-10 or TGF-β (as functional markers for Treg suppressive capacity). It would also be interesting to follow the change of this population of T cells – both in peripheral blood and atopic skin – during natural development of the disease and over the course of successful (or unsuccessful) treatment for CAD.

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Résumé

Contexte – La dermatite atopique (AD) est une maladie cutanée inflammatoire chronique fréquente de l'homme et du chien. Les cellules T régulatrices (Tregs) sont essentielles dans le contrôle de l'homéostasie immunitaire et jouent un rôle clé dans l'AD de l'homme même si la fréquence de Tregs chez les patients atopiques humains varie de façon importante. Seules deux études ont étudiées le nombre de Tregs dans le sang périphérique du chien atopique (CAD).

Objectifs – Cette étude a pour objectif d'estimer le nombre de Tregs circulants chez le chien sains et atopique et de déterminer si le nombre de Treg corrèle avec l'âge, le genre, la sévérité de la maladie ou le prétraitement.

Sujets – Des chiens de propriétaires dont 14 chiens sains et 35 chiens atopiques.

Méthodes – L'expression des cellules mononuclées Tregs dans le sang périphérique a été évaluée par cytométrie de flux. Les Tregs ont été phénotypiquement identifiés comme cellules T triplement positives pour CD4, CD25 et FoxP3.

Résultats – Le pourcentage de Tregs CD4$^+$ CD25$^+$ FoxP3$^+$ circulants chez les chiens atopiques était augmenté significativement comparé aux chiens sains (moyenne 2.1% versus 1%, $P = 0.002$) et corrélait avec la sévérité de la maladie (échelle de prurit: $r = 0.48$, $P = 0.003$; CADESI-04: $r = 0.34$, $P = 0.044$). Aucune différence significative d'âge ou de genre n'a été trouvée dans chaque groupe et le prétraitement n'avait aucune influence sur les résultats des chiens atopiques.
Conclusions – Les données suggèrent que, comme pour l'AD humaine, les Tregs CD4$^+$ CD25$^+$ FoxP3$^+$ peuvent contribuer à la pathogénie de la DAC telle que l'indique l'association entre la fréquence de Treg et la sévérité de la maladie. De plus amples investigations sont nécessaires pour améliorer la compréhension du rôle des Tregs dans la dermatite atopique.

Resumen
Introducción – La dermatitis atópica (AD) es una enfermedad crónica inflamatoria de la piel común en seres humanos y perros. Los linfocitos T reguladores (Tregs) son componentes esenciales del control de la homeostasis inmunitaria y han demostrar tener un papel clave de la dermatitis atópica humana, incluso aunque la frecuencia de los linfocitos T reguladores en los pacientes humanos con atopia varían enormemente. Sólo dos estudios han publicado los números de linfocitos T reguladores en la sangre periférica de perros con dermatitis atópica canina (CAD).
Objetivos – este estudio está enfocado a evaluar los números de linfocitos T reguladores en perros sanos y perros con atopia y determinar si los números de linfocitos T reguladores se correlacionan con la edad, sexo, severidad de la enfermedad o tratamiento previo.
Animales – perros de propietarios privados incluyendo 14 perros sanos y 35 perros con atopia.
Métodos – la expresión de linfocitos T reguladores en células mononucleares de sangre periférica se evaluó mediante citometría de flujo. Los linfocitos T reguladores fueron típicamente identificados como células triples positivas para CD4, CD25, y FoxP3.
Resultados – el porcentaje de linfocitos T reguladores CD4+, CD25+ FoxP3+ en perros atópicos se incrementó significativamente comparado con los perros sanos (media 2,1% frente a 1%, $P = 0{,}002$), y se correlacionó con la severidad de la enfermedad (escala de prurito: $r = 0{,}48$, $P = 0{,}003$; CADESI-04: $r = 0{,}34$, $P = 0{,}044$). No hubo diferencias significativas en edad basados en edad o sexo en ningún grupo y el tratamiento previo no tuvo influencia en los resultados en perros tópicos.
Conclusión e importancia clínica – los datos sugieren que, al igual que en humanos, los linfocitos T reguladores CD4+, CD25+ FoxP3+ pueden contribuir a la patogénesis de la dermatitis atópica canina tal y como indica la asociación entre la frecuencia de linfocitos T reguladores y la severidad de la enfermedad. Se requieren más investigaciones para mejorar la comprensión del papel de los linfocitos T reguladores en perros atópicos.

Zusammenfassung
Hintergrund – Die atopische Dermatitis (AD) ist eine häufige chronische entzündliche Hauterkrankung bei Menschen und Hunden. Die regulatorischen T Zellen (Tregs) sind essentielle Kontrolleure der Homöostase des Immunsystems und es konnte gezeigt werden, dass sie bei der AD des Menschen eine wichtige Schlüsselrolle spielen, obwohl die Anzahl der Tregs bei atopischen Menschen stark variieren. Es gibt bisher nur zwei Studien, die über die Anzahl der Tregs im peripheren Blut von Hunden mit caniner AD (CAD) berichten.
Ziele – Das Ziel dieser Studie war eine Erhebung der Anzahl der zirkulierenden Tregs bei gesunden und atopischen Hunden, und festzustellen, ob die Anzahl der Tregs mit dem Alter, dem Geschlecht und der Schwere der Erkrankung oder mit einer Vorbehandlung korrelierten.
Tiere – Hunde im Privatbesitz, davon 14 gesunde Hunde und 35 Hunde mit CAD.
Methoden – Die Exprimierung der Tregs in peripheren mononukleären Zellen des Blutes wurde mittels Flowzytometrie bestimmt. Die Tregs wurden phänotypisch als CD4, CD25 und FoxP3 positiv identifiziert.
Ergebnisse – Der Prozentsatz der zirkulierenden CD4$^+$CD25$^+$FoxP3$^+$ Tregs bei atopischen Hunden war im Vergleich zu gesunden Hunden signifikant erhöht (Durchschnitt 2,1% versus 1%, P=0,002) und korrelierte mit der Schwere der Erkrankung (Juckreizskala: r=0,48, P=0,003; CADESI-04: r=0,34, P=0,044). Es bestanden in beiden Gruppen keine signifikanten Unterschiede zwischen dem Alter oder dem Geschlecht, eine Vorbehandlung hatte keinen Einfluss auf die Ergebnisse bei atopischen Hunden.
Schlussfolgerungen – Die Ergebnisse weisen darauf hin, dass wie beim Menschen CD4$^+$CD25$^+$FoxP3$^+$ Tregs an der Pathogenese der CAD teilhaben, was sich durch den Zusammenhang der Anzahl der Tregs und der Schwere der Erkrankung zeigte. Eine weitere Untersuchung ist nötig, um das Verständnis der Rolle der Tregs bei atopischen Hunden zu erweitern.

要約
背景 — アトピー性皮膚炎(AD)はヒトおよびイヌの一般的な慢性炎症性皮膚疾患である。制御性T細胞(Tregs)は免疫ホメオスタシスの必須制御要素で、ヒトADにおいてTregsの頻度は患者により大きく異なっているにも関わらず、重要な役割を果たしていることが示されている。イヌAD(CAD)のイヌの末梢血におけるTregs数は2つの研究でしか報告されていない。

目的 — この研究は健康なイヌとアトピー犬における循環中Tregsの数を評価すること、およびTregs数が年齢、性別、疾患の重症度あるいは治療前と関係しているのかを明らかにすることを目的とした。
供与動物 — 14頭の健康な飼い犬と35頭のCADの飼い犬。
方法 — 末梢血の単核細胞におけるTregsの発現をフローサイトメトリーにより評価した。TregsはCD4、CD25およびFoxP3の3つが陽性のT細胞として表現型を特定した。
結果 — アトピー犬における循環中$CD4^+$ $CD25^+$ $FoxP3^+$ Tregsの割合は健常犬と比較し有意に増加し(平均2.1% 対 1%, P = 0.002)、疾患重症度と相関していた(そう痒スケール: r = 0.48, P = 0.003; CADESI-04: r = 0.34, P = 0.044)。年齢あるいは性別にはいずれの群においても有意差は認められず、アトピー犬で治療前であることは結果に影響しなかった。
結論 — データからヒトと同様、$CD4^+$ $CD25^+$ $FoxP3^+$ Tregsは、Tregs頻度および重症度の間の関係が示しているようにCADの病因の一因であるかもしれない。アトピー犬におけるTregsの役割への理解を深めるために、さらなる調査が必要とされる。

摘要
背景 — 异位性皮炎是种在人和犬上均常见的慢性炎性皮肤病。调节性T细胞(Treg)是基本的免疫稳态控制器,并已认定在人AD上起重要作用,尽管人异位性患者Treg出现的频率差异很大。只有两篇研究报告过AD患犬外周血中Treg数量。
目的 — 本次研究目的为评估健康犬和AD患犬的循环Treg数量,并确定Treg数量分别与年龄、性别、疾病严重程度或治疗是否有关。
动物 — 来自动物主人的14只健康犬和35只AD患犬。
方法 — 使用流式细胞术评估Treg在外周血单核细胞中的表达。具有CD4、CD25和FoxP3三重阳性反应表型的T细胞,被认为是Treg。
结果 — 异位性皮炎患犬循环中CD4+ CD25+ FoxP3+Tregs比健康犬显著增多(平均值2.1% 比 1%, P = 0.002),并与疾病严重度有关(瘙痒评分: r = 0.48, P = 0.003; CADESI-04: r = 0.34, P = 0.044)。其与年龄、性别没有显著关联,异位性皮炎患犬治疗后无明显改变。
结论与临床意义 — 数据显示,与人类一样,通过Treg频率和疾病的严重程度间的关联,可知CD4+CD25+FoxP3+Tregs参与CAD的发病机理。需要进一步调查,帮助我们提高对犬异位性皮炎中Treg作用的认识。

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